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In vitro anti-leishmanial activity of methanolic extracts of *Calendula officinalis* flowers, *Datura stramonium* seeds, and *Salvia officinalis* leaves

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[ABSTRACT]

AIM: The anti-leishmanial activity of methanolic extracts of *Calendula officinalis* flowers, *Datura stramonium* seeds, and *Salvia officinalis* leaves against extracellular (promastigote) and intracellular (amastigote) forms of *Leishmania major* were evaluated in this study.

METHOD: In the first stage, promastigote forms of *L. major*, were treated with different doses of the plant extracts in a 96-well tissue-culture microplate and IC_{50} values for each extract were measured with colorimetric MTT assay. In the second stage, macrophage cells were infected with *L. major* promastigotes. Infected macrophages were treated with plant extracts. Then the macrophages were stained with Gimsa and the number of infected macrophages and amastigotes were counted with a light microscope.

RESULTS: The results indicated that the plant extracts inhibited the growth of promastigotes and amastigotes of *L. major*. Inhibitory concentrations (IC_{50}) for promastigote assay were 108.19, 155.15, and 184.32 $\mu g \cdot mL^{-1}$ for *C. officinalis* flowers, *D. stramonium* seeds and *S. officinalis*, respectively. The extracts also reduced the number of amastigotes in macrophage cells from 264 for control group to 88, 97, and 102 for test groups. Although the anti-leishmanial activity of the extracts were not comparable with the standard drug, miltefosine; but they showed significant efficiency in reducing the number of amastigotes in macrophages, in comparison with the control group ($P < 0.001$). These plant extracts had lower toxicity compared with miltefosine.

CONCLUSION: This study demonstrates the potential efficacy of the methanolic extracts of *C. officinalis* flowers, *D. stramonium* seeds, and *S. officinalis* leaves to control of cutaneous leishmaniasis.

[KEY WORDS] *Leishmania major;* Promastigote*;* Amastigote*; Calendula officinalis; Datura stramonium; Salvia officinalis*

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Introduction

Cutaneous leishmaniasis (CL) is the common form of a disease caused by an obligate intracellular protozoa called leishmania, and is distinguished with cutaneous lesions on the exposed parts of the body. Although this form of leishmaniasis (CL) is not lethal and is mostly self-healing, it leaves permanent scars, which are often the cause of serious social stigmas [1-2]. The infection is acquired when a person is bitten by an infected sand fly. When leishmania first enters the human body, it is in the promastigote form. Macrophages uptake promastigotes, and are then transformed into the intracellular form of leishmania, called the amastigote [1-2]. Leishmaniasis is an endemic disease in 88 countries, especially in developing countries. Ninety % of cutaneous leishmaniasis cases occur in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia, Syria, and Iran [3]. As there is no vaccine for prevention, drug treatment (pentavalent antimony, amphotericin B,

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and miltefosine) is the only way to control leishmaniasis [4-6]. However, many of the chemical drugs have toxic effects on different organs of human body [7]. Furthermore, the development of resistant parasites to most drugs has been reported [7-8]. On the other hand, leishmaniasis is a poverty-related disease and is associated with malnutrition, poor housing, and weakness of the immune system and lack of financial resources [9]. Thus cheapness and accessibility are very important factors for drug selection. Plant materials are likely to provide a valuable, inexpensive, and available source for new medicinal agents. Adverse effects of phytotherapeutic agents are also less common compared with chemical drugs. Therefore, extracts derived from plants are suggested to have new compounds active against leishmania. However, phytopharmaceuticals can be used as a drug for treatment, only if their effectiveness versus chemical drugs is proven [9]. Plant materials, such as saponins, quinones, alkaloids, terpenes, phenol derivatives, and some other metabolites are reported to have anti-parasitic and anti-leishmanial activities [10]. Plants were used in this study have many medicinal uses. *Calendula officinalis* L. (Asteraceae) flowers (pot marigold) have anti-microbial effects [11-12] on some microorganisms such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*; and anti-fungal effects against *Candida albicans* and *Candida parapsilosis* [13-14]. There is a report about the anti-parasitic effects of oleanolic acid and glycosides isolated from *C. officinalis* [15]. Oleanolic acid was also found in *Salvia officinalis* L. (Lamiaceae) and showed antimicrobial activity [16]. Furthermore, this plant is indicated to have some anti-fungal activities against *Saccharomyces cerevisiae* [17]. The anti-microbial and anti-fungal activities of *D. stramonium* seed extract were also reported [18-19].

In this study, the anti-leishmanial activities of methanolic extracts of *C. officinalis* flowers, *Datura stramonium* L. (Solanaceae) seeds and *S. officinalis* leaves against *L. major* promastigotes, were evaluated in cell free cultures, and against amastigotes, in a macrophage cell line.

Materials and Methods

Plants materials

Plants were collected from Tehran province, Iran, in June 2011. Botanical identification was performed by botanists in the Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran, and voucher specimens were retained in the herbarium (*C. officinalis* number: 1499, *D. stramonium* number: 1677, *S. officinalis* number: 1533).

Preparation of extracts

The extraction process was performed using the cold percolation method. The *C. officinalis* flowers, *D. stramonium* seeds, and *S. officinalis* leaves were dried at room temperature, and then were ground with a blender. A sample (50 g) of each powder was macerated in 80% methanol (300 mL) for 48 h in room temperature with shaking. The crude extracts were obtained after filtration with Whatman No. 1 filter paper, and were evaporated under low pressure at below 40 °C in a rotor evaporator [20]. The extracts were dried, weighed, and finally dissolved in 1% DMSO with 31.25, 62.5, 125, 250, 500, and 1 000 μg·mL^{-1} proportions for further usage.

Parasite

L. major promastigotes (strain MROH/IR/75/IR, provided by the Pasteur Institute of Iran) were used for *in vitro* screening. The promastigotes were maintained in Novy-Macneal-Nicolle (NNN) medium, at 25 ºC. Phosphate buffered saline (PBS, pH 7.2) was used as the liquid phase of the culture medium.

Anti-promastigote activity assay

L. major promastigotes were cultured in RPMI medium supplemented with 10% fetal calf serum overnight, to reach the logarithmic phase. Then, plant extracts dissolved in DMSO were added to a 96-well tissue-culture micro plate and serial dilution, with descending concentrations, from 1 000 to 31.25 μg·mL^{-1}, was performed. Neubauer chambers containing 2 × 10^6 promastigotes/mL were then added to each well (50 μL). The plate was incubated at 25 ºC for 48 h. The viability of promastigotes was assessed by tetrazolium-dye (MTT) colorimetric method [21-22]. Briefly, MTT solution (25 μL, 0.5 mg·mL^{-1}) was added to each well and incubated for 3–4 h at 37 ºC. Then, DMSO (100 μL) was added to dissolve the MTT formazan, and finally the optical density (OD) was measured using an ELISA plate reader at 570 nm. The control wells contained DMSO without drugs. In addition, miltefosine (Sigma, USA) was used as the standard drug.

Anti-amastigote activity assay

The macrophage J774 cells (ATCC number TIB-67) were supplied by the Pasteur Institute of Iran. The cells were cultured in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum and penicillin (100 U·mL^{-1})-streptomycin (100 μg·mL^{-1}), and were maintained at 37 °C in a CO_2 incubator. The anti-amastigote activites of the plant extracts were performed according to routine methods [23]. Briefly, the cells (4 × 10^5/well) were cultured in a 24-well microplate containing glass coverslips. After 4 h, non-adherent cells were removed and plate was incubated at 37 °C and 5% CO_2 for 24 h. After incubation, macrophages were infected with *L. major* promastigotes with a parasite/macrophage ratio of 7 : 1, and after 4 h incubation, free promastigotes were removed by washing with PBS. After an overnight incubation at 37 ºC and 5% CO_2, the plant extracts were added to the plate wells with concentrations of 1 000 to 31.25 μg·mL^{-1}. The media containing drugs were renewed after 48 h. After 60 h incubation, coverslips were washed with PBS, fixed in methanol, stained with 10% Giemsa solution, and examined with a light microscope. The number of infected macrophages and also amastigotes, in 100 macrophages, were counted and the results presented as infection rate (IR) and parasite survival (PS) [22]:

IR = No. of infected macrophages in 100 macrophages

PS=

$$\frac{\text{No.of amastigotes inexperimental culture/100 macrophages}}{\text{No.of amastigotes in control culture/100 macrophages}}\times 100$$

Cytotoxicity for macrophages

The toxicity of extracts for macrophage cells was assessed by the MTT colorimetric assay. First, the J774 macrophage cells (2×10^5/well) were added in a 96 wells micro plate. After 24 h incubation at 37 ºC and 5% CO_2, the cells were exposed to plant extracts at the same concentrations that were used in the anti-amastigote assay. After 60 h incubation at 37 ºC, MTT (20 μL, 0.5 mg·mL^{-1}) were added to each well, and then the plate was re-incubated for 4 h. The optical density (OD) at 570 nm was measured. Cell viability was determined using the following formula [22]:

$$\text{Cell viability (\%)} = \frac{\text{Optical density in experimental well}}{\text{Optical density in control well}}\times 100$$

Statistical analysis

Data are presented as $\bar{x} \pm s$ of the mean (SEM). Differences between groups were analyzed by one-way ANOVA followed by Tukey multiple comparison test. A $P < 0.05$ was considered to be statistically significant

RESULTS

Anti-promastigote assay

Results of this study demonstrated that IC_{50} value for methanolic extracts of *C. officinalis* flowers, *D. stramonium* seeds, and *S. officinalis* leaves in the promastigote assay were at doses of 108.19, 155.15, and 184.32 μg·mL^{-1}, respectively (Table 1). Miltefosine, as a standard drug, with an IC_{50} value of 5.3 μg·mL^{-1} showed a potent anti-leishmanial activity on the *L. major* promastigotes. The IC_{50} value for the miltefosine group was significantly less than those of the plant extracts ($P < 0.001$).

Table 1 IC50 values for methanolic plant extracts in promastigote assay.

Groups	Family	Organ	Common name	IC_{50} for promastigote assay (μg·mL^{-1})
C. officinalis	Asteraceae	Flowers	Pot marigold	108.19 ± 8.6
D. stramonium	Solanaceae	Seeds	Jimson weed	155.15 ± 10.1
S. officinalis	Lamiaceae	Leaves	garden sage	184.32 ± 11.7
Miltefosine	—	—	—	5.3 ± 1.2**

** $P < 0.001$ *vs* other groups

L. major promastigotes treated with the *C. officinalis* extract had the lowest viability among the other extracts.

Anti-amastigote assay

Different concentrations of the plant extracts were tested for their activity against amastigotes in macrophage cells. Infection Rate (IR) values demonstrate the percentage of infected macrophages in samples. IR values showed a significant decrease in test groups compared with the control group ($P < 0.001$). This decrease suggests that some macrophages were cleaned from amastigotes (Fig. 1). Miltefosine, with twenty infected macrophages after treatment, had a better anti-amastigote activity than other groups. IR value for *C. officinalis* extract, was significantly lower than that of the *S. officinalis* group ($P < 0.05$), and also than *D. stramonium* extract. However, the parasite survival (PS) is a factor which better shows the effects of the extracts on amastigotes compared to IR, since it can represent the extent of infection. The PS values in the amastigote assay were 33%, 40%, and 42% for *C. officinalis* (108.19 μg·mL^{-1}), *D. stramonium* (155.15 μg·mL^{-1}), and *S. officinalis* (184.32 μg·mL^{-1}), respectively (Table 2). The number of *L. major* amastigotes in macrophages treated with plant extracts were significantly lower ($P < 0.001$) than the negative control. There was no significant difference ($P > 0.05$) between the number of *L. major* amastigotes in macrophages treated with different plant extracts (Fig. 2). Miltefosine, with more than 80% amastigote proliferation inhibitory activity (Table 2) and fifty-five amastigotes in one hundred macrophages (Fig. 2) had the best anti-amastigote activity among other groups.

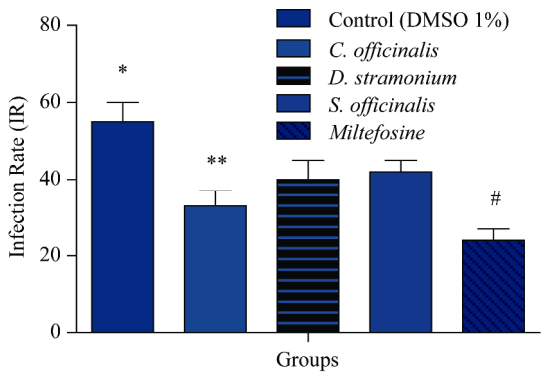

Fig. 1 The efficacy of methanolic plant extracts of macrophages infected with *L. major* amastigotes. ** $P < 0.001$ *vs* control group

Table 2 The efficacy of methanolic plant extracts on parasite survival (PS) and toxicity for a macrophage cell line infected with *L. major* amastigotes

Groups	Control	*C. officinalis* (108.19 μg·mL^{-1})	*D. stramonium* (155.15 μg·mL^{-1})	*S. officinalis* (184.32 μg·mL^{-1})	Miltefosine (5.3 μg·mL^{-1})
Parasite survival (PS) (%)	96 ± 6.2	33 ± 2.8*	40 ± 3.3*	42 ± 3*	20 ± 1.7*
Toxicity for macrophage cell line (%)	0	10 ± 1.3	12 ± 1.4	8 ± 0.8	22 ± 2**

* $P < 0.01$ *vs* control group; ** $P < 0.001$ *vs C. officinalis*, *D. stramonium*, and *S. officinalis* groups

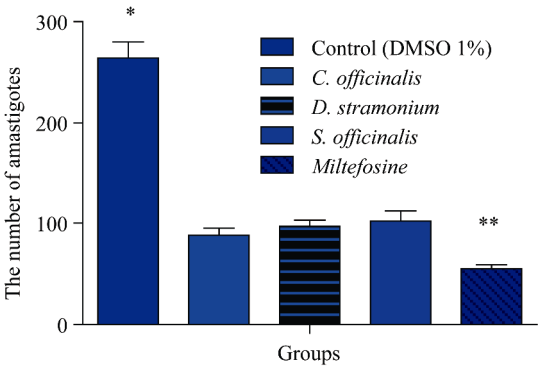

Fig. 2 Efficacy of methanolic plant extracts on the number of amastigotes in 100 macrophages. ** $P < 0.001$ *vs* control group

Among the plant extracts, the flowers of *C. officinalis* had the best anti-leishmanial activity on both forms of leishmania. The other two plant extracts also had acceptable activity (IC_{50} below 200 μg·mL^{-1}) against *L. major* promastigotes and amastigotes.

Cytotoxicity to macrophages

The cytotoxicity of *C. officinalis* flowers, *D. stramonium* seeds and *S. officinalis* leaves methanolic extracts were determined at IC_{50} concentration for each extract (Table 2). The cytotoxicity of these extracts were lower than ($P < 0.001$) that of miltefosine. The *S. officinalis* extract had the lowest toxicity against the macrophage cell line with only 8% toxicity at the IC_{50} concentration (184.32 μg·mL^{-1}), whereas miltefosine had the highest toxicity (22%) at 5.3 μg·mL^{-1} (Table 2).

DMSO did not show any activity against *L. major* promastigotes and amastigotes (up to 1% in complete medium).

Discussion

Cutaneous leishmaniasis is one of the most common forms of the disease. It usually produces ulcers on the exposed parts of the body. Although leishmaniasis is mostly self-healing without any treatment, it can cause serious debilitating problems [1, 24]. The routine treatments for leishmaniasis, pentavalent antimonial drugs, amphotericine B, and miltefosine, can cause serious side effects. Drug resistance has also become an important problem. Therefore, identification and development of new medicinal agents is an urgent need for alternative treatment [7, 25]. On the other hand, most of the endemic areas are developing countries that have limited accessibility to effective drugs [3]. For this reason, it is necessary to discover endemic plants with anti-leishmanial activities; since plants can provide a valuable, safe, and inexpensive source of anti-microbial agents. Numerous studies from all over the world have been performed to recognize natural products with anti-leishmanial effects [26-28]. In the present study, methanolic extracts of *C. officinalis* flowers, *D. stramonium* seeds, and *S. officinalis* leaves showed considerable inhibitory effects against leishmania promastigotes with IC_{50} values below 0.2 mg·mL^{-1}, and against amastigotes with more than 60% lethality of parasite internalized to macrophages. Findings in this study indicate that methanolic extracts of *C. officinalis* flowers have high anti-leishmanial activity. However, the IC_{50} value of this extract against *L. major* promastigotes (108.19 μg·mL^{-1}) was much higher than that of miltefosine (5.3 μg·mL^{-1}), a well-known drug for leishmaniasis. The efficacy of the *C. officinalis* flowers extract on leishmaniasis is supported by the anti-amastigote activity of this extract on infected macrophages. The plant extract significantly reduced the number of amastigotes (88 amastigotes in the sample wells, compared to 264 amastigotes in the negative control wells).

The anti-promastigote and anti-amastigote activities of *D. stramonium* seeds and *S. officinalis* leaves were lower than that of the *C. officinalis* flower extract. However, both extracts reduced the infectivity of *L. major* amastigotes in macrophages, similar to *C. officinalis*. Although anti-leishmanial activities of these plant extracts are not comparable with the standard drug (miltefosine), but they show potential efficacy for treatment of leishmaniasis. Therefore this could be a primary step in the preparation of natural drugs for cutaneous leishmaniasis. The plants used in this study are known to have antibacterial [11, 16, 18] and antifungal [12, 17, 19] properties, but there were no reports of antileishmanial activity. Some compounds known as antiparasitic [10], such as alkaloids, saponins, triterpenes, and flavonoids were found in these plant extracts [13, 29-31]. Therefore, it is likely that the anti-leishmanial activity of these plant extracts is performed by similar compounds. However, further studies and fractionation of the plant extracts, are necessary to determine the components responsible for anti-leishmanial activity.

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